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Development of a Sensitive and Rapid Chromogenic Factor IX Assay for Clinical Use

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Key Words. Chromogenic assay · Factor VIII purification · Factor IX · Factor X activation

Abstract. A chromogenic factor IX assay is developed which requires only two time-dependent steps. Diluted plasma is mixed with a reagent containing factors VIII and X. The reaction is started by addition of a reagent containing factor XI_a, thrombin, $CaCl_2$, and phospholipids. Then factor XI_a activates factor IX if present, thrombin activates factor VIII, and subsequently the complete factor X activating complex (factor IX_a, factor $VIII_a$, Ca ions, and phospholipids) rapidly activates factor X. Finally, ethylenediaminetetraacetic acid plus a chromogenic substrate are added to stop the reaction and to measure formed factor X_a. Factor X_a formation is proportional to the plasma factor IX concentration (from 0 to 140%). The two reagents needed for the assay are stable at room temperature during a whole working day and for 3 h at 37 °C. A new isolation procedure for factor VIII is described. Factor VIII is purified from bovine plasma in a few steps with a yield of 20% and a 8,000-fold purification.

Introduction

Factor IX (Christmas factor) is an important zymogen of the blood coagulation. Deficiency of this protein in blood leads to bleeding problems: hemophilia B [see ref. 1 for a review]. Factor IX is a vitamin K dependent protein and thus contains γ-carboxyglutamic acid residues. These γ-carboxyglutamic acid residues are possibly involved in the binding of factor IX (and also other vitamin K dependent clotting factors) to negatively charged phospholipid membranes via calcium bridges [2]. Human factor IX is a glycoprotein with a carbohydrate content of about 17% and with an apparent molecular weight of 57,000 [3, 4]. The plasma concentration is 46-88 n*M*. Factor IX can be activated by factor XI_a and by the factor VII_a-tissue factor complex. Both enzymes cleave the same bonds in factor IX which results in a two-chain factor $IX_a\beta$ and an activation peptide (Mr = 11,000).

Factor IX_a is an important enzyme of the intrinsic pathway of blood coagulation [for reviews see refs. 5 and 6]. Factor IX_a activates factor X in a reaction that is accelerated enormously by factor $VIII_a$, phospholipids, and calcium ions [7]. Several methods are described to measure factor IX_a: a clotting assay using factor IX deficient plasma [8], a method based on the esterase activity of factor IX_a using radiolabeled esters [9], a method to determine the release of the tritiated activation peptide which originally was described by Silverberg et al. [10], an immunoradiometric assay [11], a spectrometric assay described by Tans et al. [12], and an active-site titration with *p*-nitrophenyl-*p*-guanidino benzoate hydrochloride [13]. All these methods (except the clotting assay which, however, is not very precise) have the disadvantage of being laborious and thus are unsuited for a routine determination in a clinical laboratory.

In order to measure factor IX quantitatively, (1) it has to be activated, and (2) the formed factor IX_a should activate factor X under conditions that factor IX_a is the rate-limiting compound. In this paper we searched for optimal reaction conditions and a procedure to minimize the incubation time and the pipetting steps. For a chromogenic factor IX assay we do not need a 100% pure factor VIII; however, the preparation should not contain fibrinogen or other components that interfere with the assay. In this paper we describe the isolation of bovine factor VIII
describe the isolation of bovine factor VIII
that meets the conditions for a well-working factor IX assay.

Materials and Methods

Materials

FX_a substrate (CH_3OCO-*D*-CHG-Gly-Arg-*p*NA-AcOH) and α-NAPAP [N-α-(2-naphthylsulfonylglycyl)-*D,L*-amidinophenyl-alanine-piperidide hydroidide] were obtained from Pentapharm (Switzerland); Sphérosil X 015 LS was from Rhône Poulenc (France); Sephadex G-25 fine was from Pharmacia (Sweden); immunoadsorbed factor IX deficient plasma was donated by Baxter Dade (Switzerland); S2337 [N-benzoyl-*L*-isoleucyl-*L*-glutamyl-(piperidyl)-*L*-glycyl-*L*-arginine-*p*-nitroanilide hydrochloride], S2238 (H-*D*-phenylalanyl-*L*-pipecolyl-*L*-arginine-*p*-nitroanilide dihydrochloride) and RVV-X (factor X activator from Russell's viper venom) were from Kabi (Sweden).

All other reagents were of the highest grade commercially available.

Phospholipids and Phospholipid Vesicles

Phosphatidylcholine was extracted from egg yolks according to the method of Bligh and Dyer [14] and purified as described by Comfurius and Zwaal [15]. Phosphatidylserine was prepared in a similar way, however, extracted from bovine brains. Vesicles were prepared by sonication of a phospholipid mixture in a buffer containing 50 m*M* Tris-HCl and 175 m*M* NaCl (pH 7.9) using an MSE Mark II 150-watt ultrasonic disintegrator set at 9 μ peak-to-peak amplitude. The composition of the vesicles was 75 mol% phosphatidylcholine and 25 mol% phosphatidylserine or as indicated.

Proteins

All proteins were isolated from bovine plasma. Factor X was isolated according to the method of Fujikawa et al. [16]. Human factor IX [see refs. 17 and 18] was a gift of T. Janssen-Claessen. Factor IX_a was prepared by activating human factor IX with isolated factor XI_a according to the method of Fujikawa et al. [19]. Bovine factor XI_a (contact product) was isolated as described by Nossel [20] and Østerud and Rapaport [21]. Prothrombin was isolated according to the method of Owen et al. [22]. Thrombin was prepared from prothrombin by activation with prothrombinase as described elsewhere [23].

Factor VIII was measured as described by Wagen-
Factor VIII was measured as described by Wagen-
voord et al. [24]. As reference plasma normal pooled citrated plasma was used that was

healthy donors; the plasma was donated by T. Janssen-Claessen. The reference plasma was compared a few times with the reference plasma provided by Baxter (CoagCal). Both plasmas contained the same amount of factor VIII; differences of less than 3% were found. By definition, normal pooled plasma contains 1 U/ml factor VIII.

To measure factor X_a formation in time, samples of the reaction mixture were added to a cuvette containing 20 m*M* ethylenediaminetetraacetic acid in standard buffer (see below). The final volume after subsampling was 980 µl. Factor X_a then was measured by addition of 20 µl of 4 m*M* FX_a substrate plus 50 µ*M* α-NAPAP to the cuvette [see ref. 24]. α-NAPAP was added to the FX_a substrate to inhibit hydrolysis of this chromogenic substrate by thrombin. 1 µ*M* α-NAPAP inhibits thrombin by 99.5% [24].

Reaction Conditions

All reactions were done at 37 °C; in each case the reagents were preincubated at 37 °C during 5 min. All proteins were dissolved in 175 m*M* NaCl and 50 m*M* Tris-HCl (standard buffer). All dilutions were done in the same buffer containing 0.5 mg/ml ovalbumin.

Results

Isolation of Factor VIII

Bovine blood was collected in 10-liter vessels containing 1 liter 100 m*M* benzamidine, 100 m*M* sodium citrate, 20 U/ml heparin, and 100 mg/l soybean trypsin inhibitor. The next steps were done at 0–4 °C. The blood was centrifuged at 5,000 *g* during 15 min. The plasma was collected and to the plasma was added polyethylene glycol 20,000 (1.8%, w/v). The mixture was stirred during 10 min, and then the formed precipitation was spun down at 2,000 *g* (10 min). The precipitate was dissolved in 120 m*M* NaCl and 10 m*M* trisodium citrate (final volume about one twentieth of the plasma volume). To the solution was added 50 m*M* β-mercaptoethanol, and the mixture was incubated during the night at 0 °C. Next day the formed precipitation was spun down at 5,000 *g* during 30 min. The supernatant usually was frozen at –80 °C until further use.

A few factor VIII preparations were thawed and combined. To the preparation was added Sphérosil PL (4 g/l). Sphérosil PL was prepared by addition of 5% (w/w) phospholipids dissolved in chloroform (10 mg/ml) to Sphérosil (X 015 LS). The phospholipid composition was 95 mol% phosphatidylcholine and 5 mol% phosphatidylserine. The chloroform was evaporated in vacuum until dryness. Then the Sphérosil PL was washed with isotonic salt. The factor VIII preparation was stirred during the night with the Sphérosil PL at room temperature. Next day the Sphérosil PL was collected and poured into a small column (5 cm^2). The Sphérosil PL was washed very carefully with isotonic salt. Factor VIII was eluted from Sphérosil PL with 1 *M* sodium thiocyanate and 1 *M* NaCl (pH 6.2). The eluted factor VIII was gel filtrated immediately to remove the salt by elution on a Sephadex G-25 column (50 × 5 cm^3) which was equilibrated with 175 m*M* NaCl and 50 m*M* Tris-HCl (pH 7.9). The activity of factor VIII varied from 100 to 250 U/ml. The optical density at 280 nm of the preparations was about 1. The obtained preparation did not promote platelet aggregation, so it is free of active von Willebrand factor.

Properties of Factor VIII

We have determined the amount of thrombin that is needed for complete activation of factor VIII. In figure 1 the result of this experiment is shown. One can observe that remarkably high concentrations of thrombin (more than 100 n*M*) are necessary to activate factor VIII completely.

The amount of activated factor VIII necessary for complete saturation of the factor IX_a was determined in a similar way as described by Van Dieijen et al. [25]. In a mixture containing human factor IX_a, $CaCl_2$, phospholipid vesicles, thrombin, and factor X, the factor VIII concentration was varied (1.25–10 U/ml). In this reaction mixture, the factor X activating reaction was linear, with the reaction time between 15 and 90 s. The reaction rates were plotted double reciprocally against the factor VIII concentrations, obtaining straight lines from which the kinetic constants of the reaction were calculated. The V_{max} is 4.256 n*M*/min, and the turnover number is 851 (moles factor X_a formed per minute per mole factor IX_a). To obtain a half maximal reaction rate, 3.64 U/ml factor VIII is required. This means that very high factor VIII concentrations are necessary to saturate the factor X activating complex almost completely. For a 90% saturation, already 32.8 U/ml factor VIII is required. By increasing the final $CaCl_2$ concentration to 13.4 m*M*, the V_{max} was hardly changed (792); however, the amount of factor VIII necessary for a half maximal rate increased tenfold (32.8 U/ml). This means that by increasing the $CaCl_2$ concentration, the affinity of factor $VIII_a$ to the factor X activating complex decreases. So it is possible to modulate the reaction rate by changing the $CaCl_2$ concentration.

In the same way as described by Van Dieijen et al. [25] we have determined the molar concentration of 1 U/ml factor VIII. At a very low factor VIII concentration (1/120 U/ml), the assay was saturated with excess of factor IX_a. We found that the dissociation constant of factor IX_a for the factor X activating complex was 17.3 n*M*, and the maximal rate of factor X_a formation was 4.88 n*M*/min. From these data we have calculated that 1 U/ml factor VIII equals 0.688 n*M*, assuming that factors $VIII_a$ and IX_a are present in the factor X activating complex at equimolar amounts [25].

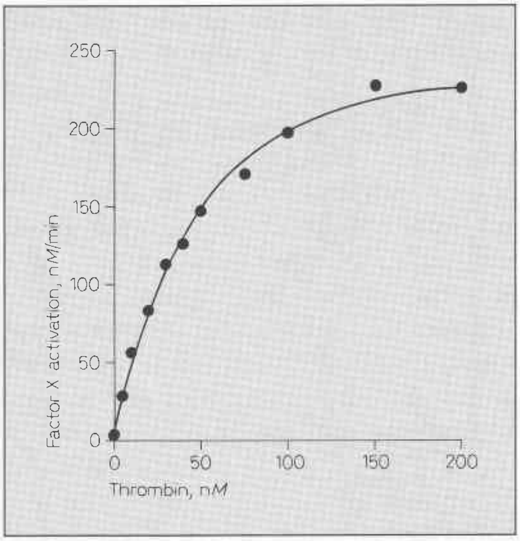

Fig. 1. Activation of factor VIII as a function of the thrombin concentration. Factor VIII (5 U/ml) was activated with thrombin during 15 s in the presence of 5 m*M* $CaCl_2$. Forty microliters of the activated factor VIII was added to 160 µl of 1.25 µ*M* factor X, 25 µ*M* phospholipid vesicles, 5 m*M* $CaCl_2$, and 25 n*M* factor IX_a. Samples of 20 µl were taken at 20 and 40 s reaction time, and the factor X_a formation was measured.

The results described above have shown that for practical reasons it is not possible to work with saturating amounts of factor VIII; this means that the rate of factor X_a formation depends on the factor VIII concentration in the reaction mixture. For that reason we have studied the stability of the factor VIII at 37 °C. Figure 2 shows that factor VIII is not stable in the absence of $CaCl_2$; however, when 0.1 m*M* $CaCl_2$ is present, factor VIII is stable. The activity in the absence

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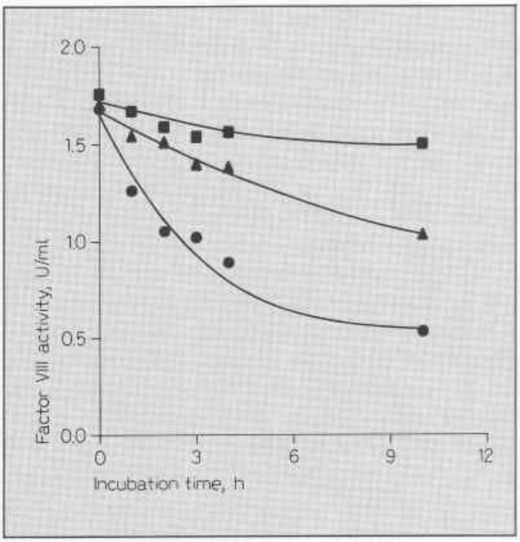

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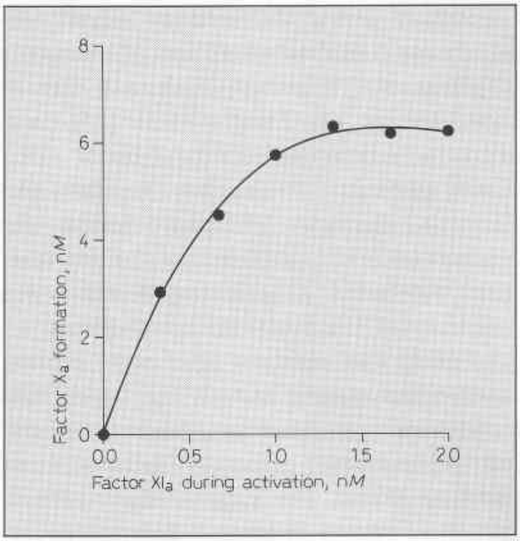

Fig. 2. Stability of diluted factor VIII at 37 °C. Factor VIII was diluted to about 1.7 U/ml in standard buffer containing 2.0 mg/ml ovalbumin and no (●), 25 μ*M* (▲), or 100 μ*M* (■) $CaCl_2$.

Fig. 3. Activation of human factor IX with factor XI_a. To 100 μl 400 n*M* thrombin, 80 μ*M* phospholipid vesicles, and 15 m*M* $CaCl_2$ was added 100 μl factor XI_a of 0, 1, 2, 3, 4, 5, and 6 n*M*. At t = 0 min 100 μl human factor IX (50 p*M*) was added and at t = 2 min 100 μl of a mixture containing 2.5 U/ml factor VIII, 1 μ*M* factor X, and 5 m*M* $CaCl_2$. At t = 4 min samples of 200 μl were taken, and the formed factor X_a was measured. To correct for hydrolysis of FX_a substrate by factor XI_a, the same experiment was done without factor X.

of $CaCl_2$ dropped by 68% after 10 h at 37 °C, whereas in the presence of 0.1 m*M* $CaCl_2$ the activity dropped by only 20%. So, by addition of 0.1 m*M* $CaCl_2$ to the reagent with factor VIII, it is sufficiently stable to use in the chromogenic factor IX assay.

Optimization of the Factor IX Assay

We have determined the optimal concentrations of factor XI_a, phospholipid vesicles, and factor X for a well-working chromogenic factor IX assay.

In figure 3 we have determined the concentration of factor XI_a that is necessary to activate human factor IX completely within 2 min. This concentration is about 1.0 n*M*.

The dependency of the assay on phospholipid concentration and composition is very similar as described by Van Dieijen et al. [7] and Wagenvoord et al. [24]. For that reason only the results of these studies are given. The reaction rate is maximal at a phospholipid concentration of 8 μ*M* (25 mol% phosphatidylserine, 75 mol% phosphatidylcholine). At higher concentrations the activity slowly decreases, and at lower concentration it sharply drops to almost zero in the absence of phospholipids. We have also studied whether changing the composition of the phospholipids affects the reaction rate. Addition of cholesterol or phosphatidylethanolamine to the vesicles did not lead to higher

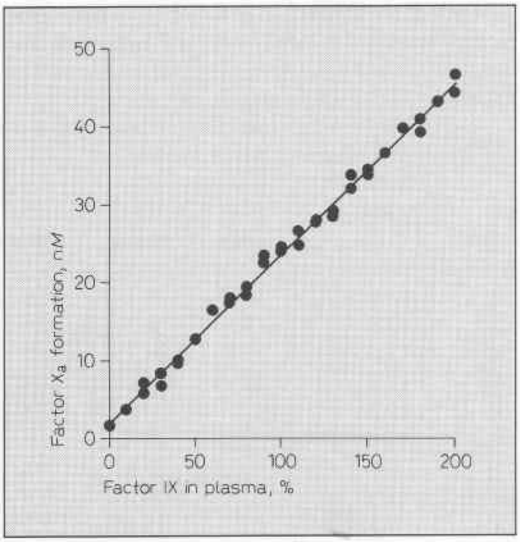

Fig. 4. Factor IX assay of plasmas with variable amounts of factor IX. Immunoadsorbed factor IX deficient plasma was 200 times diluted in standard buffer (0% factor IX), normal pooled plasma was 200 times diluted (100% factor IX) and 100 times diluted (200% plasma) in the same buffer. By mixing the diluted plasmas, we prepared plasmas with 0–200% factor IX. The pipetting sequence was: at t = 0 min 100 µl reagent 1 (300 n*M* thrombin, 15 m*M* $CaCl_2$, 2 n*M* factor XI_a, 60 µ*M* phospholipid) was mixed with 100 µl diluted plasma; at t = 2 min 100 µl reagent 2 (1 µ*M* factor X, 3 U/ml factor VIII, 0.1 m*M* $CaCl_2$) was added, and at t = 4 min samples of 100 µl were taken to measure formed factor X_a.

reaction rates, and changing the ratio between phosphatidylcholine and phosphatidylserine from 10 to 2 in the vesicles did not lead to higher activities.

In a similar way as described by Wagenvoord et al. [24], the K_m of factor X activation by the complete factor X activating complex was determined. The K_m is 45 n*M*. The K_m is comparable with that found in the case of factor VIII assay [24]. Although in that case the composition of the factor X activating complex was different, it was composed of bovine factor IX_a and human factor $VIII_a$.

The results described above have shown that an optimal factor IX assay is obtained when the factor XI_a concentration is 1 n*M* during factor IX activation, the thrombin concentration during factor VIII activation is 100 n*M* or more, and the final factor X concentration is far above the K_m (300 n*M*). We decided to use 20 µ*M* phospholipids because at this concentration the reaction is not very much dependent on the phospholipid concentration.

It was shown that very high factor VIII concentrations are required to bind all factor IX_a in the factor X activating complex. For practical reasons it is not possible to use such high amounts of factor VIII on large scale. For that reason we have investigated whether the formed factor X_a is linearly dependent on the factor IX concentration under conditions that factor VIII is not saturating.

Properties of the Factor IX Assay

To minimize pipetting steps we have prepared two reagents with compositions based on the above-described results. Reagent 1 contained 300 n*M* thrombin, 15 m*M* $CaCl_2$, 2 n*M* factor XI_a, and 60 µ*M* phospholipid vesicles. Reagent 2 contained 1 µ*M* factor X, 3 U/ml factor VIII, and 0.1 m*M* $CaCl_2$. The $CaCl_2$ in reagent 2 is added to stabilize the factor VIII. The generally used pipetting sequence was: at t = 0 min 100 µl of reagent 1 was mixed with 100 µl diluted plasma, at t = 2 min 100 µl of reagent 2 was added, and at t = 4 min samples of 100 µl were taken to measure formed factor X_a.

Figure 4 shows that the assay is linear with the percentage factor IX in the plasma

Table 1. Stability of the factor IX assay reagents

Incubation		Activity	
time, h	temperature °C	factor X_a nM/min	%
0	22	27.3	100
4	22	25.9	94.9
24	22	25.2	92.6
0	37	27.3	100
1	37	26.9	98.5
3	37	25.7	94.4

Method and composition of the reagents 1 and 2 are described in text. At the indicated incubation time, a fresh plasma dilution was made (normal pooled plasma, 200 times diluted).

up to 200%, although the factor VIII concentration is 1 U/ml which is below the K_d.

Because we use nonsaturating factor VIII concentrations in the factor IX assay, the assay is sensitive for inactivation of factor VIII. The other clotting factors are present in excess, so changes in their concentration would not affect the reaction rate very much. We have prepared the reagents 1 and 2 and tested whether they are stable at 37 °C and at room temperature. In table 1 the results of these tests are shown. One can notice that even after 3 h at 37 °C or after 24 h at 22 °C (ambient temperature) the assay is stable within 7.4%.

Factor IX Detection in Different Plasmas with Modified Reagents

For clinical application of the chromogenic factor IX assay, plasma dilutions of 1 in 200 are not practical. In clinics one would like to dilute the plasma in one step. Plasma dilutions of 1 in 31 or 1 in 41 are more suitable. However, the reaction rate would be far too high when the reaction conditions shown in figure 4 are used. As was described above, the reaction rate can be decreased by increasing the $CaCl_2$ concentration of the reaction mixture. The reaction rates can be decreased also by addition of smaller plasma volumes to the reaction mixture. For those reasons we prepared modified reagents 1 and 2 and

Table 2. Determination of factor IX in plasmas of patients treated chronically with oral anticoagulants

Plasma	Factor IX in plasma, %			
	chromogenic factor IX assay	APTT method		
		5 times	10 times	100 times
1	14.6	24.6	21.8	12.8
2	13.8	23.3	20.5	11.5
3	13.6	25.5	21.8	11.7
4	12.3	22.9	20.2	11.3
5	19.2		30.3	15.9
6	23.4		29.4	17.4
7	20.6		27.1	14.6
8	15.0		20.0	12.8
9	9.5		15.8	7.3
10	12.9		17.5	7.4
11	14.6		21.4	10.5
12	17.6		27.1	14.2
13	24.2		35.4	18.3
14	11.6		20.2	11.3
15	26.6		32.9	17.7
16	16.3		26.9	15.4

Factor IX was measured in two ways: either using the modified chromogenic factor IX assay described in figure 5b or the standard APTT method using factor IX deficient plasma; as 100% we used either five times, ten times, or 20 times diluted reference plasma (CoagCal, Baxter). In the last case the plasma samples were diluted 100 times, and corrections were made to account for the additional dilution.

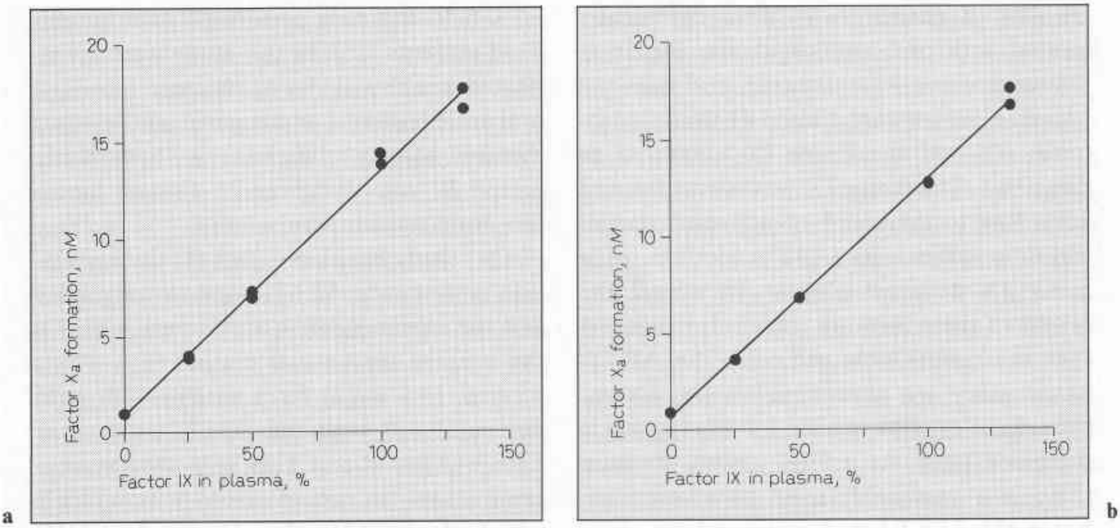

Fig. 5. Standard curves for the chromogenic factor IX assay. Two modified reagents were prepared. The modified reagent 1 contained 225 n*M* thrombin, 45 μ*M* phospholipid vesicles, 30 m*M* $CaCl_2$, and 1.25 n*M* factor XI_a. The modified reagent 2 contained 1.5 U/ml factor VIII, 750 n*M* factor X, and 0.1 m*M* $CaCl_2$. Normal pooled plasma was diluted in isotonic salt 31, 41, 82, and 164 times. For the blank isotonic salt was used. The plasma dilution 1 in 41 is called 100%. **a** The pipetting scheme was: at t = 0 min 100 μl reagent 1 was mixed with 25 μl diluted plasma, and at t = 3 min 100 μl reagent 2 was added. At t = 4.5 min 500 μl stop buffer (i.e., 20 m*M* ethylenediamine-tetraacetic acid, 128 μ*M* FX_a substrate, 1.6 μ*M* α-NAPAP) was added, and factor X_a formation was immediately measured. The final $CaCl_2$ concentration in the reaction mixture was 13.4 m*M*. **b** 100 μl reagent 2 was mixed with 25 μl diluted plasma. At t = 0 min 100 μl reagent 1 was added to start the reaction. After 3 min the reaction was terminated by addition of 500 μl stop buffer, and the formed factor X_a was immediately measured.

changed the pipetting sequence somewhat (see legend to figure 5). By having present FX_a substrate in the stop buffer, factor X_a was measured immediately, and one subsampling step was avoided. By mixing reagent 2 with the diluted plasma, starting the reaction with reagent 1 and terminating the reaction after 3 min by addition of stop buffer, the procedure was simplified even more.

Figure 5a shows a reference curve of factor IX determinations in human plasma with the modified chromogenic factor IX assay. Normal pooled plasma was diluted as indicated. A dilution of 1 in 41 is called 100% factor IX. One can observe that also in this case a linear relationship exists between the percentage factor IX and the factor X_a formation. The blank value is higher than in figure 4 because the whole reaction mixture is used. The blank is caused by hydrolysis of the chromogenic substrate by factor XI_a. In figure 5b the reference curve is shown for the most simplified procedure, i.e., starting the reaction with reagent 1. Also in this case the percentage factor IX is proportional with the factor X_a formation.

Using the method described in the legend to figure 5, we have measured factor IX in plasmas of patients who were chronically treated with oral anticoagulants. Firstly, a reference curve was prepared, and then the patient plasmas were diluted 41 times in isotonic salt and the factor IX measured as described. The factor IX was also measured according to the standard activated partial thromboplastin time (APTT) method using factor IX deficient plasma. In table 2 the results of these tests are shown. It is noticed that the results obtained with the APTT-based assay are dependent on the plasma dilution. A possible reason for this finding is discussed later. At a high plasma dilution there is a reasonable good correlation between the APTT-based assay and the chromogenic factor IX assay.

We also have investigated stability and activity of the modified reagents after lyophilization. Modified reagents 1 and 2 were prepared, divided in 2-ml amounts, and lyophilized. Their activity was tested before and after lyophilization. Before lyophilization with a plasma dilution of 1 in 41 and isotonic salt (blank), the formed amounts of factor X_a were 13.76 and 0.97 n*M*, respectively. After lyophilization, these values were 13.74 and 1.25 n*M*. After 6 months, these levels were not decreased: 13.99 and 1.08 n*M*, respectively.

The sensitivity of the assay is determined in plasma. In ten determinations in reference plasma we found 14.15 ± 0.419 n*M*/min, whereas for 24 blank determinations values of 0.545 ± 0.053 n*M*/min were found. A variation of about 10% is found for the blank. A 1% plasma gives a rate of 0.136 n*M*/min in addition to the blank which is 2.5 times the standard deviation. So it is well possible to measure an amount of 1% plasma; however, to be precise, the measurement must be repeated a few times.

A field trial was performed with lyophilized reagents [26]. In this study were investigated healthy subjects, patients receiving oral anticoagulants and heparin, and various diseases, namely mild and severe hemophilia A and B, von Willebrand's disease, lupus-like anticoagulant, and others.

Our study has shown that (1) the correlation between the APTT-based clotting assay and the chromogenic assay is very well; (2) the average for normal plasma (n = 40) is 95.4 ± 14.0%; (3) for a patient with mild hemophilia B with the APTT-based assay 13% was found and 11% with the chromogenic assay – in case of severe hemophilia B with both assays less than 1% was found; (4) in cases of mild and severe hemophilia A with both assays similar results were found: 90 and 93%, 66.5 and 62%, 50 and 56.5%, and 62 and 72%, respectively – also in case of von Willebrand's disease the results were similar: 80 and 84 and 74 and 80%, respectively, and (5) tests with factor IX concentrate showed a good correlation: with the APTT-based assay were found 20.9, 21.0, and 26.7 U/ml and with the chromogenic assay 23.1, 19.6, and 25.6 U/ml.

Discussion

In this paper we have described the development of a chromogenic factor IX assay that is well suited for clinical use. Tans et al. [12] already described a spectrophotometric assay for human plasma factor IX. The main difference between the assay described by Tans et al. [12] and our assay is the use of isolated purified factor VIII. For that reason the assay described by these authors requires a much longer time to activate an appreciable amount of factor X. Another difference between both assays is the time needed for factor IX activation. Tans et al. [12] use a separate preactivation step of 25 min, whereas in our case factor XI is activated in the final mixture within a few minutes. This shows that in spite of the accuracy, the method of Tans et al. [12] is not suitable for clinical use, because the procedure takes a long time, and a number of pipetting steps are necessary. The procedure described in this article is simple and requires only two pipetting steps at fixed times. The reagents necessary for the assay can be kept at room temperature during a whole working day without loosing their activity. Because of the simplicity of the method, it is well possible to automate the whole procedure. The lyophilized reagents ready for use can be obtained from Baxter.

Because we need large amounts of purified factor VIII for the factor IX assay, it was necessary to develop a new isolation procedure for bovine factor VIII. The kinetic properties of this factor VIII are comparable with those described for bovine facor VIII [25] which was isolated in a different way [27]. The removal of von Willebrand factor from the preparation can occur during two steps in the isolation procedure. During reduction of the preparation, von Willebrand factor likely will be destroyed [27] and the interaction of factor VIII with von Willebrand factor possibly is broken during binding of factor VIII to Sphérosil PL. Andersson and Brown [28] showed that this interaction is broken when the complex binds to phospholipid vesicles.

Remarkable is the finding that the factor IX assay is linear with the amount of factor IX, even at nonsaturating amounts of factor VIII. This means that under the described conditions the fraction of factor IX_a that is bound in the factor X activating complex fully depends on the factor $VIII_a$ concentration and is not dependent on the factor IX_a concentration. The factor $VIII_a$ concentration in the assay mixture is 1 U/ml which is about 0.7 n*M*. The reaction rate is about 10 n*M*/min in case of the plasma with 100% factor IX. Assuming a turnover rate of 851, the factor X activating complex concentration is 12 p*M*. This amount is only 1.7% of the total factor $VIII_a$ concentration, so the change in free factor $VIII_a$ concentration by doubling the factor IX_a concentration (at most) is only 1.7%. These changes will not have any measurable effect on the fraction of factor IX_a that will be bound, and thus the assay will be linear over the whole range of the assay. The plasma concentration of factor IX is about 60 n*M*, so the concentration of factor IX_a in the assay mixture is about 100 p*M* (fig. 4). This means that only 12% of the factor IX_a is bound in the complete factor X activating complex. In case of figure 5, this percentage is even lower (about 2%) because of the high $CaCl_2$ concentration.

It was shown that by increasing the $CaCl_2$ concentration in the reaction mixture, the formation of the complete factor X activating complex is inhibited. At a concentration of 13.4 m*M* $CaCl_2$, the activation of factor X by factor IX_a in the absence of factor $VIII_a$ is almost optimal [7], and also activation of factor IX by factor XI_a probably is not slower than at 5 m*M* $CaCl_2$ [29, 30]. So the inhibiting effect of a high concentration of $CaCl_2$ is only noticed when factor $VIII_a$ is present. The mechanism of this inhibition might be decreased binding of factor $VIII_a$ to the complete factor X activating complex at a higher $CaCl_2$ concentration. This phenomenon requires further investigation, but this falls outside the scope of this article.

The finding that increasing the $CaCl_2$ concentration decreases the activity of the complete factor X activating complex was used to develop an assay suitable for clinical use. Having present 13.4 m*M* $CaCl_2$ (final concentration) in the reaction mixture, the reaction is inhibited so much that it is not necessary to dilute plasma 200-fold, but only 41-fold. The plasma sample is diluted 41 times, so the final plasma dilution in the reaction mixture is 369 times. This means that clotting factors in the plasma are diluted so that they do not affect the assay, whereas inhibitors of activated clotting factors also cannot have a detectable effect on the assay. When plasma is used that is anticoagulated with 3.8% sodium citrate, the citrate concentration in plasma will be about 10 m*M* and thus in the reaction mixture 0.027 m*M*. This citrate will bind Ca ions, however, less than 0.2%, so a detectable increase of the reaction rate (because less Ca ions are present) cannot be expected.

The lyophilization experiments have shown that the reagents 1 and 2 can be lyophilized without loss in activity. The lyophilized reagents can be stored for at least a few months without loosing their activity. These results together with the stability tests show that the assay is well suited for practical clinical use.

We have measured factor IX in different types of plasma, namely normal pooled plasma, factor IX deficient plasma, plasmas with a known percentage of factor IX (prepared by mixing normal pooled plasma with factor IX deficient plasma), and plasmas of patients who were treated chronically with oral anticoagulants. When we measured factor IX in plasmas of known factor IX concentrations, there was a good correlation between the theoretical amount of factor IX and the results found in the assay. The assay is not sensitive for other factors than factor IX; for that reason, the plasma dilution is not important. The signal is halved either when the plasma is diluted twice or when it contains 50% factor IX. Tran et al. [26] showed that the correlation between the APTT-based assay and the chromogenic assay is very well. In 1 case discrepancies were found, testing plasmas from patients receiving oral anticoagulants. The discrepancies were due to the APTT-based test system. Tran et al. [26] also showed that the amount of measured factor IX was dependent on the plasma dilution in case of the APTT test. The more the plasma was diluted, the lower percentage factor IX was detected in the plasma, whereas with the chromogenic assay at every dilution, the same percentage was found. At very high plasma dilutions the APTT gives the same result as the chromogenic assay.

We have found the same phenomenon also. In table 2 this is illustrated: the more the plasma is diluted, the lower percentage factor IX is measured. Table 2 also shows that the correlation is reasonable between the chromogenic assay and the APTT-based assay, provided that the plasma is sufficiently diluted. The reason why the result of the APTT-based assay depends on the plasma dilution still has to be determined, but this falls outside the goal of this article. It might be related to the behavior of the decarboxylated clotting factors which possibly are present in plasma of patients treated with oral anticoagulants. These oral anticoagulants (warfarin derivatives) inhibit the carboxylation (formation of γ-carboxyglutamic acid residues) of the vitamin K dependent clotting factors (factors IX, X, VII, and II). The decarboxylated clotting factors possibly show some activity when their concentration is high (thus at a low plasma dilution), but at lower concentrations they have no activity, because they have a low affinity to phospholipid membranes.

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